Plant Physiol. (1979) 64, 472–475
0032-0889/79/64/0472/04/$00.50/0

Fatty Acids and Circadian Rhythms in *Phaseolus coccineus*

EFFECTS OF LIGHT, TEMPERATURE, AND CHEMICALS[1]

Received for publication November 28, 1977 and in revised form July 18, 1978

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ABSTRACT

Five major fatty acids, palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3), were identified in polar lipid extracts from pulvini of *Samanea saman* and *Phaseolus coccineus*. In *P. coccineus* their distribution varied quantitatively in the laminar pulvinus, petiolar pulvinus, petiole, stem, leaf and root. Short pulses of red light did not greatly affect the relative quantities of fatty acids in dark grown *P. coccineus*, but a 30-minute exposure of red light generally increased the degree of unsaturation by increasing linolenic acid and decreasing linoleic and palmitic acids.

***P. coccineus* seeds were exposed to several substituted pyridazinones as well as cerulenin and dimethylethanolamine. The pyridazinones San 6706 and norflurazon altered fatty acid composition but also altered morphology and inhibited chlorophyll synthesis. Exposure to 10 C for 72 hours caused a small but significant increase in the degree of unsaturation of *P. coccineus* fatty acids but results were equivocal with *S. saman*.**

Recent evidence indicates that membrane-based phenomena may be involved in circadian rhythmicity. Njus and co-workers (9) and Sweeney (15) have proposed hypotheses involving membranes and transport processes as a basis for circadian rhythms. The theory of Njus and co-workers (9) proposes that the fatty acid content of cell membranes plays a regulatory role in the circadian rhythm and in the process of temperature compensation of period length. This is based on the observation that many poikilothermic organisms change their fatty acid composition in response to external temperature, the degree of unsaturation of fatty acids increasing as temperature is lowered and vice versa (1–3, 14).

We have investigated the fatty acid content of the pulvinus of *Phaseolus coccineus* to determine if fatty acids play a role in the well established circadian rhythm of this plant. The fatty acid content of *Samanea saman*, another plant whose leaves undergo circadian oscillations, was determined and compared to the fatty acid content of *P. coccineus*. This paper reports experiments undertaken to define the fatty acid parameters in *P. coccineus*, and describes methodology of extraction and analysis and effects of light and temperature variations on the fatty acid content of polar lipids extracted from *P. coccineus* pulvini. In addition, chemicals that alter plant fatty acid composition were applied to *P. coccineus* to determine their effects on pulvinar fatty acids and rhythmic phenomena. A paper in preparation will report our investigations of possible circadian oscillations of *P. coccineus* pulvinar fatty acids.

MATERIALS AND METHODS

Plants Used and Growth Conditions. *S. saman* (Jacq.) Merrill and *P. coccineus* cv. Scarlett Runner were used for fatty acid analysis. *Samanea* plants were grown in the greenhouse in flower pots and were generally used at the age of 6 months to 1 year. *P. coccineus* seeds were soaked for 6 to 8 h in distilled H_2O before sowing in coarse washed Vermiculite in base-perforated plastic flats (50 cm long × 40 cm wide × 12 cm deep). These were kept on automatic watering tables where they were subirrigated with a solution of 1.5 g/l Hyponex plant food (Hydroponic Chemical Co., Copley, Ohio). Plants were grown in growth chambers on cycles of 12 h light followed by 12 h of darkness (LD 12:12).[5] Illumination of about 2,000 ft-c was from cool-white fluorescent lamps supplemented with about 10% incandescent. Temperature was 25 C and relative humidity was 60 ± 10%.

Lipids. Lipids were extracted from pulvini by a slight modification of the technique of Kates (5). Tissue was ground in sand and organic solvent with mortar and pestle. The first grinding was done in hot isopropyl alcohol (to inactivate lipases) +0.01% (w/v) butylated hydroxytoluene (BHT) as an antioxidant. The homogenate from this extraction was suction filtered and the filter residue reground in a mixture of chloroform-isopropyl alcohol (1:1). Chloroform was kept over CaH_2 as a desiccant. The second homogenate was suction-filtered and the filter residue washed with chloroform. Combined filtrates were then vacuum-evaporated at 38 C. Lipid was redissolved in chloroform and washed with 1% (w/v) NaCl in water to remove water-soluble impurities. The organic solvent layer was then vacuum-evaporated and the lipid redissolved in chloroform and stored in glass-stoppered fully filled volumetric flasks in the dark at −20 C until fractionation. The separation of neutral from polar lipids was accomplished by the method of Penny and Stowe (11). The lipid dissolved in chloroform was added to 100-mesh silicic acid powder. Chloroform was added and stirred to produce a slurry from which the neutral lipids were removed by suction filtration. The polar lipids were eluted from the silicic acid by washing with methanol. The methanol extract was vacuum-evaporated at 38 C, the polar lipids redissolved in methanol, and similarly stored at −20 C until methylation.

Methylation was done by the technique of Metcalfe *et al.* (7) using BF_3-methanol. Fatty acid esters were extracted from the BF_3-methanol solution with petroleum ether (b.p. 30–60 C). Petroleum ether extracts were evaporated under N_2 to a small volume and this concentrated extract of fatty acid esters was used for gas chromatography.

A Perkin-Elmer F-11 gas chromatograph with a flame ioniza-

[1] This investigation was supported by National Institutes of Health Training Grant USPHS HD 00032.

[2] This paper is based on a dissertation submitted to fulfill in part the requirements for the degree of Doctor of Philosophy at Yale University.

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[5] Abbreviations: LD 12:12: 12 h of light followed by 12 h of darkness; BF_3: boron trifluoride; DME: *N,N*-dimethylethanolamine.

tion detector was used. The column was stainless steel (183 × 0.318 cm) packed with GCM-056 12% stabilized DEGS on Anakrom ABS 90/100 mesh, purchased from Analabs, Inc. Oven temperature was 200 C and N_2 was used as the carrier gas at a rate of 5 ml/min.

Identification of Fatty Acids. Fatty acids were identified by comparing the retention of their methyl esters with those of commercial standards supplied by Analabs Inc. Individual standards were also added to experimental extracts and the corresponding peaks were observed to increase in size. Peak areas were calculated by multiplying height × width at half-height. The ratio of saturated to unsaturated was calculated by relative amounts of 16:0 + 18:0/18:1 + 18:2 + 18:3.

Temperature Treatments. *S. saman* and *P. coccineus* plants were placed in temperature-controlled incubators in darkness at the desired temperature and samples taken at selected times over the period of the experiment.

Light Treatments. Etiolated *P. coccineus* plants approximately 10 days old were used. Leaves were excised in the dark and placed on moist filter paper in Petri dishes. The red light source consisted of daylight fluorescent tubes covered with several layers of red cellophane, giving an irradiance of approximately 1200 ergs cm^{-2} s^{-1} at 600 to 690 nm. Those samples exposed were at a distance of approximately 10 cm for the appropriate time periods. Control samples, excised at the same time were kept in darkness throughout. Twenty-four h after excision, leaves and pulvini were subjected to the usual extraction procedure after having been incubated in darkness.

Chemical Treatments. Five substituted pyridazinones were used: pyrazon[5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone, San 9785 [4-chloro-5-(dimethylamino)-2-phenyl-3(2H) pyridazinone], San 9774 [5-amino-4-chloro-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone], San 6706 [4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H) pyridazinone], and norflurazon [4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H) pyridazinone]. Pyrazon was the gift of the BASF-Wyandotte Corp. of Parsippany, N.J. San 9785, San 9774, San 6706, and norflurazon were gifts of J. St. John of the USDA at Beltsville, Md. The Sandoz Wander Corp. of Homestead, Fla. also supplied larger quantities of norflurazon.

Appropriate quantities of pyridazinones for the desired concentration were dissolved in 1 ml of acetone which was added to 500 ml of distilled H_2O. Seeds were soaked in this solution for approximately 6 to 8 h. Control seeds were soaked in 500 ml of water to which 1 ml of acetone was added.

DME was supplied by the Aldrich Chemical Co. as a 99% solution. It was diluted with water to obtain the desired concentration. Cerulenin was a gift of S. Omura of Kitasato University of Tokyo, Japan. A stock solution of 2 mg/ml was made up to the desired concentration in 500 ml of water and seeds were soaked in the solutions for 6 to 8 h. Seeds which had been soaked in the various chemicals were sown in Vermiculite in plastic flats and the Vermiculite drenched with the remainder of the soaking solution. Plants were harvested for analysis 2 weeks later.

RESULTS

Five major fatty acids are present in pulvini from *Samanea* and *Phaseolus*. These five are: palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3). Table I shows the relative amounts of pulvinar fatty acids in the polar lipid fraction of *S. saman* and *P. coccineus*. The secondary pulvinus of *S. saman* and the laminar pulvinus of *P. coccineus* were analyzed. Each individual acid is expressed as a decimal fraction of the total acids in the extract. Stearic and oleic acids are present in greater quantity in *S. saman* than in *P. coccineus*. In some experiments, linoleic exceeded linolenic in quantity in *S. saman*, while in *P. coccineus*, linoleic was always present in smaller quantity than linolenic.

Anatomical Distribution of Fatty Acids in *P. coccineus*. The fatty acid contents of the other tissues in the plant were compared to that of the laminar pulvinus (pulvinus attaching the leaf blade to the petiole). Table II shows the results as an average of two experiments. The leaf blade shows the greatest difference from the laminar pulvinus with almost 80% of its fatty acid present as linolenic acid, while the root has less linolenic and proportionally more linoleic and palmitic than the laminar pulvinus.

Effects of Light. The fatty acid content of the polar lipids of etiolated plant tissue was compared with that of green tissue and with etiolated tissue given a red light exposure. The anatomy of etiolated *P. coccineus* plants is very different from that of light-grown plants. The pulvinus attaching the leaf to the petiole is extremely small in the etiolated plant and therefore the petiolar pulvinus (attaching the stem to the petiole) was used instead. Table III shows the effect of light on fatty acid content and on the saturated to unsaturated ratio in various parts of the leaf. At least a 30-min exposure to red light, followed by a dark incubation for 24 h before lipid extraction, was necessary in order to obtain any difference between red-treated and dark tissues. Dark controls were incubated for 24 h in the dark without any light treatment. Green plants were grown under standard LD 12:12 cycles for approximately 2 weeks before lipid extraction.

Compared with etiolated plants, all three tissues of light-grown plants are higher in linolenic acid, lower in linoleic and palmitic acids, and have a greater degree of unsaturation. In the pulvinus, red-treated plants show a slight increase in unsaturation compared with dark controls. Contrary to the other light treatments in the leaf, red light seems to decrease linolenic, increase linoleic and palmitic, and decrease the amount of unsaturation.

Table I. *Pulvinar Fatty Acids in the Polar Lipid Fraction of the Laminar Pulvinus of P. coccineus and the Secondary Pulvinus of S. saman*

P. coccineus plants were grown for 2 weeks in LD 12:12 cycles and *S. saman* were grown in a greenhouse and transferred to LD 12:12 cycles at least 2 weeks before extraction. Means of five replicate samples of about 20 pulvini each are expressed as a fraction of the total ± SD.

Plant	Palmitic	Stearic	Oleic	Linoleic	Linolenic
P. coccineus	0.248 ± 0.027	0.046 ± 0.004	0.059 ± 0.003	0.117 ± 0.003	0.490 ± 0.029
S. saman	0.192 ± 0.010	0.110 ± 0.011	0.044 ± 0.055	0.244 ± 0.013	0.406 ± 0.006

Table II. *Distribution of Fatty Acids from Polar Lipids in Different Regions of P. coccineus*

Means expressed as a fraction of the total ± SD. SD cited only as an indication of the range since two determinations were made.

Tissue	Palmitic	Stearic	Oleic	Linoleic	Linolenic
Laminar pulvinus	0.237 ± 0.006	0.031 ± 0.003	0.029 ± 0.005	0.151 ± 0.028	0.549 ± 0.024
Petiolar pulvinus	0.263 ± 0.002	0.045 ± 0.001	0.039 ± 0.008	0.176 ± 0.022	0.473 ± 0.013
Petiole	0.250 ± 0.027	0.031 ± 0.005	0.031 ± 0.001	0.240 ± 0.012	0.445 ± 0.018
Stem	0.228 ± 0.017	0.029 ± 0.004	0.021 ± 0.005	0.251 ± 0.053	0.386 ± 0.036
Leaf	0.103 ± 0.023	0.022 ± 0.003	0.013 ± 0.005	0.074 ± 0.011	0.792 ± 0.004
Root	0.315 ± 0.026	0.033 ± 0.001	0.041 ± 0.006	0.243 ± 0.001	0.367 ± 0.037

Temperature Experiments. Experiments were performed to determine whether pulvinar fatty acids responded to changes in temperature. Initial studies with *S. saman* indicated substantial variability in fatty acid ratios between individual plants. Subsequent experiments were done exclusively with *P. coccineus*. Two-week-old *P. coccineus* plants were incubated in the dark at 10 and 25 C for 72 h. An examination of the row labeled Avg. ± SD in Table IV indicates small but significant differences in stearic, linolenic, and the saturated to unsaturated ratio at the two temperatures. Relative to values at 25 C, stearic acid (18:0) seems to decrease, linolenic acid (18:3) appears to increase, and the saturated to unsaturated ratio appears to decrease at 10 C. These results are in agreement with the general observation (1–3, 14) that low temperature increases fatty acid unsaturation. We cannot conclude from our results that changes in fatty acid composition are occurring rapidly enough (*i.e.* within 1 day) to account for temperature compensation. However, evidence to be reported in a paper in preparation indicates that circadian rhythms in levels of some of these fatty acids may complicate the interpretation of these results.

Chemical Probes. If fatty acid content is related to rhythmic phenomena in plants, it might be possible to alter rhythmic leaflet oscillations by altering the pulvinar fatty acid content. To test this, several different chemicals which have been reported to affect fatty acid composition were applied to *P. coccineus*. St. John (13) found that substituted pyridazinones could alter the fatty acid composition of the galactolipids of wheat (*Triticum aestivum*). Five pyridazinones were tested for their effect on *P. coccineus* pulvinar fatty acids: pyrazon, San 9785, San 9774, San 6706 and norflurazon. In addition, Waring and Laties (17) reported that the antibiotic cerulenin and the choline analog DME affected phospholipid synthesis. These substances were thus applied to *P. coccineus*. San 9785 and 9774 produced no morphological effects; similarly, cerulenin and DME at concentrations of 30 μM and 1 mM, respectively, had no effect on the ratios of pulvinar fatty acids, the degree of unsaturation or the morphology of *P. coccineus* plants.

Table III. *Effect of Light on Individual Fatty Acids from Polar Lipids and Their Saturation Level in the Pulvinus, Leaf, and Petiole of P. coccineus*

Acids expressed as fraction of total from one determination.

Tissue, Light Conditions	Palmitic	Stearic	Oleic	Linoleic	Linolenic	Saturated/ Unsaturated
Petiolar pulvinus, dark	0.292	0.041	0.035	0.260	0.370	0.500
Petiolar pulvinus, 30-min red	0.260	0.034	0.036	0.261	0.408	0.417
Petiolar pulvinus, light-grown	0.263	0.045	0.039	0.176	0.473	0.447
Leaf, dark	0.148	0.017	0.014	0.114	0.707	0.197
Leaf, 30-min red	0.213	0.026	0.008	0.132	0.619	0.314
Leaf, light-grown	0.103	0.022	0.013	0.074	0.792	0.142
Petiole, dark	0.278	0.030	0.047	0.366	0.277	0.446
Petiole, light-grown	0.250	0.031	0.031	0.240	0.445	0.392

Table IV. *Effect of Temperature on Fatty Acid Content and Saturation Level of Pulvinar Polar Lipids*

Results of a 72-h incubation of 2-week-old *P. coccineus* plants at two different temperatures (10 and 25 C). Replicate samples removed for analysis at times indicated. Acids expressed as fraction of total.

Treatment	Palmitic	Stearic	Oleic	Linoleic	Linolenic	Saturated/ Unsaturated
Initial values	0.266	0.053	0.037	0.141	0.502	0.469
25 C, 12 h	0.274	0.056	0.039	0.135	0.495	0.493
25 C, 22 h	0.268	0.058	0.030	0.143	0.498	0.485
25 C, 46 h	0.286	0.053	0.027	0.135	0.496	0.515
25 C, 70 h	0.286	0.056	0.049	0.110	0.497	0.521
25 C, avg.	0.279	0.056	0.036	0.131	0.497	0.504
± SD	±0.009	±0.002	±0.010	±0.014	±0.001	±0.017
10 C, 13 h	0.265	0.051	0.042	0.131	0.512	0.461
10 C, 23 h	0.251	0.040	0.030	0.144	0.535	0.410
10 C, 47 h	0.274	0.044	0.033	0.143	0.504	0.467
10 C, 71 h	0.259	0.044	0.039	0.145	0.511	0.435
10 C, avg.	0.262	0.044	0.036	0.141	0.516	0.433
± SD	±0.010	±0.005	±0.005	±0.007	±0.013	±0.026

Table V. *Effect of Pyrazon and Norflurazon on Fatty Acids and Their Saturation Level in Polar Lipids of P. coccineus Pulvini*

Acids expressed as fractions of total. Seeds were soaked 6 to 8 h in the solutions indicated. Pulvini were harvested after 21 days in growth chamber at 25 C.

Treatment	Palmitic	Stearic	Oleic	Linoleic	Linolenic	Saturated/ Unsaturated
Untreated control	0.238	0.039	0.033	0.168	0.519	0.384
Pyrazon 0.1 mM	0.241	0.043	0.030	0.153	0.531	0.397
Pyrazon 0.3 mM	0.235	0.038	0.025	0.147	0.555	0.375
Pyrazon 1.0 mM	0.262	0.039	0.059	0.147	0.509	0.420
Norflurazon 0.1 mM	0.252	0.047	0.043	0.255	0.401	0.427
Norflurazon 0.3 mM	0.271	0.051	0.053	0.239	0.387	0.474
Norflurazon 1.0 mM	0.278	0.052	0.043	0.223	0.403	0.494

Pulvinar fatty acids from plants exposed to San 9785 and San 9774 showed no significant difference from the control plants, unlike the results of St. John (13). San 6706 and norflurazon significantly increased palmitic acid, while almost totally inhibiting Chl accumulation and leaf development. Table V shows a dose-response experiment for pyrazon and norflurazon. Although there is no great difference between fatty acids of control and pyrazon-treated plants, 1.0 mM pyrazon appears to produce a slight decrease in the amount of unsaturated fatty acids with a corresponding increase in the saturated to unsaturated ratio. Norflurazon at all concentrations increases the amount of saturated acids and decreases the amount of unsaturated acids with a resulting increase in the saturated to unsaturated ratio.

DISCUSSION

These experiments lay the foundation for an investigation of the possible role of fatty acids in plant circadian rhythms to be described in a paper in preparation.

As in previous investigations with other plants (8, 16), *P. coccineus* plants exposed to light have a higher degree of unsaturation in their fatty acids than do etiolated plants. The effect of a 30-min red light pulse on leaves, shown in Table III, departed from this expected pattern, but a light pulse followed by a return to darkness may initiate different metabolic processes in leaves than a continuous exposure to light. Lörcher (6) has reported experiments indicating that a red light pulse can initiate a leaf movement rhythm in arrhythmic, etiolated *P. coccineus* plants.

In most poikilothermic organisms, the degree of unsaturation of fatty acids tends to increase as the temperature is lowered and decrease as the temperature is raised (1–3, 14). Since unsaturated fatty acids generally have lower melting points than saturated fatty acids, this could represent an adaptation to changes in the environment. In plants, changes in fatty acid saturation level in response to temperature have been studied with respect to cold hardiness. Gerloff *et al.* (3) demonstrated an approximate doubling of the fatty acid content of alfalfa roots during hardening, due to the preferential accumulation of unsaturated fatty acids, particularly linolenic, while St. John (14) found that fatty acid

composition of polar lipids in cotton root tips became more unsaturated as temperature decreased from 30 to 15 C. Hitchcock and Nichols (4) concluded that the O_2 available to the desaturases is a major control mechanism in unsaturated fatty acid biosynthesis in plants. Decreasing the temperature increases O_2 solubility in water, resulting in greater desaturase activity.

Njus and co-workers (9) have proposed that changes in the saturation level of membrane fatty acids with a resultant change in membrane fluidity may be the mechanism of temperature compensation in circadian rhythms. To support such a theory, it would be necessary to demonstrate changes in fatty acid composition in a rhythmic system in response to temperature within a relatively short time. The exposures of *P. coccineus* plants to different temperatures summarized in Table IV were undertaken to determine if changes in fatty acid composition occurred rapidly enough to account for temperature compensation. Although a significant increase in fatty acid unsaturation occurred by 3 days, we cannot conclude from Table IV that significant changes are occurring by 1 day. The experiments may be complicated by rhythmic oscillations of fatty acids to be reported in a paper in preparation. This could account for some of the variability in Table IV. A conclusive test for rapid adaptation of fatty acids to low temperature would have to eliminate the complicating factor of possible rhythmic changes in saturation level. In other systems, temperature adaptation of lipids occurs fairly rapidly: approximately 3 h in the earthworm *Lampito mauritii* (12), 2 h in *Tetrahymena* (18), and 0.5 h in *Escherichia coli* (10).

San 9785- and San 9774-treated plants showed no significant difference in fatty acid content from control plants. This is different from the results of St. John (14) who showed that these pyridazinones had significant effects on the fatty acid content of wheat. In her experiments, pyridazinones were impregnated into filter paper, the wheat seeds germinated on the filter paper, and lipids extracted from 4-day-old lyophilized shoot tissue. The methodology and tissues assayed were quite different from those reported here. San 6706 and norflurazon altered the fatty acid content in the expected way, but these compounds caused morphological alterations of the plants so that observations of leaf movements could not be made. The application of compounds which alter lipid and fatty acid metabolism coupled with studies of the overt rhythm (*e.g.* leaf movement in plants) might be one way of testing the applicability of a membrane hypothesis of circadian rhythms in plants.

Acknowledgments—We thank A. W. Galston for constant experimental advice and for criticism of the manuscript. R. L. Satter also helped frame certain experimental approaches.

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